1. Title: New tools for diet analyses: nanopore sequencing of metagenomic DNA from stomach contents to quantify diet in an invasive population of rats

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6. Running title: Quantifying rat diets by direct nanopore sequencing

Abstract

Introduced species of mammals in New Zealand have had catastrophic effects on populations of diverse native species. Quantifying the diets of these omnivorous and predatory species is critical for understanding which native species are most impacted, and to prioritize which mammal species and locations should be targeted with control programmes. A variety of methods have been applied to quantify diet components in animals, including visual inspection of gut contents (Daniel 1973; Pierce and Boyle 1991), stable isotope analysis (Major et al. 2007; Carreon-Martinez and Heath 2010), and time-lapse video (Brown and Brown 1997; Dunlap and Pawlik 1996). Increasingly, DNA-based metabarcoding methods are being used (King et al. 2008; Soininen et al. 2009). These metabarcoding methods require a PCR step using primers that bind to highly conserved genomic regions (e.g. mitochondrial COI) to amplify specific regions for sequencing. This step introduces significant bias, primarily due to the lack of a universal primer set (King et al. 2008). Here we show that direct metagenomic sequencing using the Oxford Nanopore Minion allows rapid quantification of rat diets. Using a sample of rats collected from within 100km of Auckland, NZ, we show that these rats consume a wide variety of plant, invertebrate, vertebrate, and fungal taxa, with substantial differences in diet content between locales. We then show that, based on diet content alone, it is possible to pinpoint the sampling location of an individual rat within tens of kilometres. We expect that the rapidly increasing accuracy and throughput of nanopore-based sequencing, as well as increases in the species diversity of genomic databases, will soon allow rapid and unbiased assessments of animal diets in field settings.

Introduction

The relatively recent introduction of mammalian species to New Zealand has had significant negative effects on many of the native animal populations, including insects (Gibbs 1998), reptiles (Towns, Daugherty, and Cree 2001), molluscs (Stringer et al. 2003), and birds (Diamond and Veitch 1981; Dowding and Murphy 2001). These mammalian predators include possums, rats, stoats, and hedgehogs, as well as domestic pets such as cats (Gillies and Clout 2003). Currently, an ambitious plan is being put into place that aims for the eradication of all mammalian predators by 2050 (http://www.doc.govt.nz/predator-free-2050; Russell et al. 2015). A useful step toward this goal would be to prioritise the management of predators and geographical areas in which native species are experiencing the highest levels of predation. To do so requires establishing the diet content of these predators.

Unbiased and sensitive assessment of diet content is extremely difficult to achieve due to the limited accuracy of available methods. Identification of prey items using visual examination of stomach contents is strongly affected by which items are most easily degraded (for example, soft-bodied species). Stable isotope analysis is imprecise, yielding only broad information on diet such as relative consumption of protein and plant matter, as well as information on whether prey items are terrestrial or marine in origin (Hobson 1987; Basha et al. 2016). Time-lapse video (Brown et al. 2008) requires identification of the specific prey item, often difficult or impossible for small prey items or in low-light conditions.

Perhaps the most widely applied current molecular method is DNA metabarcoding. This approach relies on the PCR amplification and DNA sequencing of conserved regions from nuclear, mitochondrial, and/or plastid genomes (King et al. 2008). With adequate primer selection, this method can detect a wide range of species, and does not require specific expertise necessary for other methods (for example identifying degraded prey items).

However, DNA metabarcoding is not free from bias. PCR primers must be specifically tailored to particular sets of taxa or species (Jarman et al. 2002). Although more "universal" PCR primer pairs have been developed (for example targeting all bilaterians or even all eukaryotes; (Jarman, Deagle, and Gales 2004), all primer sets exhibit bias towards certain taxa. Tedersoo et al. (Tedersoo et al. 2015) found five-fold differences in fungal operational taxonomic units (OTU) estimates when using different sets of fungal-specific PCR primer pairs. Leray et al. (Leray et al. 2013) found that published universal primer pairs (i.e. those that do not target specific taxa) were capable of amplifying only between 57% and 91% of tested metazoan species, with as few as 33% of species in some phyla being amplified at all (e.g. cnidarians). Deagle et al. argued that in general, COI regions are simply not sufficiently conserved, and thus should not be used for metabarcoding studies at all (Deagle et al. 2014). Finally, Pawluczyk et al. showed that different loci from the same species exhibit up to 2,000-fold differences in qPCR-estimated DNA quantity within samples (Pawluczyk et al. 2015). It has even been shown that the polymerase itself can bias diversity metrics when using metabarcoding methods (Pereira et al. 2018). For these reasons, a less biased molecular-based method is desirable.

Metagenomic approaches, in which all DNA in the sample is directly sequenced, offer a promising alternative. Such methods have been used for diet analysis in only very few and recent instances (Srivathsan et al. 2015; Paula et al. 2015; Srivathsan et al. 2016). Although metagenomic approaches decrease the bias arising from PCR amplification of specific DNA regions, additional biases can arise, as the presence or absence of species and genera can only be inferred for those species or genera present in genomic databases. Although this is similarly true for metabarcoding approaches, metabarcode databases are considerably more comprehensive in terms of species representation as compared to genomic databases. Importantly, genomic sequence databases are rapidly increasing in species diversity, as are the methods to query these large databases (Wood and Salzberg 2014; Kim et al. 2016).

Here, we quantify rat diet composition using a metagenomic approach based on nanopore sequencing (Oxford Nanopore Technologies). The data suggest that rat diets around Auckland, New Zealand, comprise a wide range of plant, animal, and fungal families. We show using multivariate analyses that rat diets are distinguishable by habitat and location. This work establishes long-read metagenomic methods as a straightforward approach for diet quantification.

Materials and Methods

Study Areas

We trapped rats from three locations near Auckland, New Zealand. Each location comprised a different type of habitat: undisturbed inland native forest (Waitakere Regional Parklands, WP); native bush surrounding an estuary (Okura Bush Walkway, OB); and restored coastal wetland (Long Bay Regional Park, LB) (**Fig. 1**). Traps in OB and LB were baited with peanut butter, apple, and cinnamon wax pellets; or bacon fat and flax pellets. Traps in WP were baited with chicken eggs, rabbit meat, or cinnamon scented poison pellets. From 16 November to 16 December 2016, traps were surveyed by established volunteer conservation groups at each site every 48 hours. A total of 36 rats were collected from these locations. The majority of rats collected (34/36) were determined to be male by visual inspection, and identified as *Rattus norvegicus*. These 34 rats were selected for further analysis.

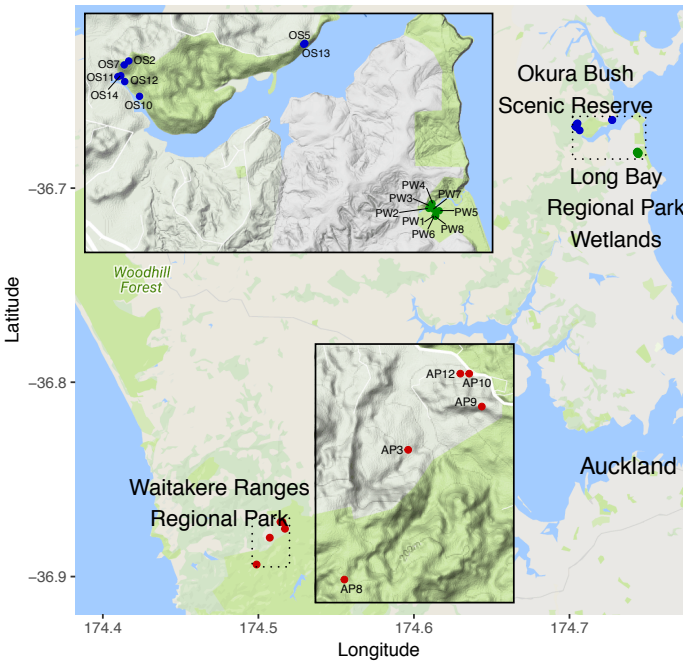

Fig. 1. Location of rat sampling sites in the greater Auckland area in the North Island of New Zealand. Each point indicates a trap where one rat was captured, with the colour of the points indicating the three broad locations: the native estuarine bush habitat of Okura Bush (OB), the restored wetland of Long Bay (LB), and the native forest of Waitakere Park (WP). The two insets show the three locations in higher resolution with topographical details. Green indicates park areas. Precise geographical coordinates were only available for five out of eight rats in WP.

DNA Isolation

Within 48 hours of trapping, rats were stored at either -20°C or -80°C until dissection. We removed intact stomachs from each animal, rinsed them with 70% ethanol, and removed the contents. After snap freezing in liquid nitrogen, we homogenised the stomach contents using a sterile mini blender to ensure sampling was representative of the entire stomach.

125 We purified DNA from 10-20 mg of homogenised stomach contents using the Promega
126 Wizard Genomic DNA Purification Kit, with the following modifications to the Animal
127 Tissue protocol: after protein precipitation, we transferred the supernatant to a new tube
128 and centrifuged a second time to minimise protein carryover. The DNA pellet was
129 washed twice with ethanol. These modifications were performed to improved DNA
130 purity. We rehydrated precipitated DNA by incubating overnight in molecular biology
131 grade water at 4°C and stored the DNA at -20°C. DNA quantity, purity, and quality was
132 ascertained by nanodrop and agarose gel. The DNA samples were ranked according
133 quantity and purity (based on A260/A280 and secondarily, A230/A280 ratios). The eight
134 highest quality DNA samples from each location were selected for DNA sequencing.

135 DNA Sequencing

136 Sequencing was performed on two different dates (24 January 2017 and 17 March
137 2017) using a MinION Mk1B device and R9.4 chemistry. For each sequencing run, DNA
138 from each rat was barcoded using the 1D Native Barcoding Kit (Barcode expansion kit
139 EXP-NBD103 with sequencing kit SQK-LSK108) following the manufacturer's
140 instructions. Twelve samples were pooled and run on each flow cell, for a total of 24
141 individual rats. The flow cells had 1373 active pores (January) and 1439 active pores
142 (March). Sequencing was performed using local base calling in MinKnow v1.3.25
143 (January) or MinKnow v1.5.5 (March), but both runs were re-basecalled after data
144 collection using Albacore 2.2.7 with the following command: *read_fast5_basecaller.py -i*
145 *./fast5_input -t 48 -s basecalled_output -f FLO-MIN106 -k SQK-LSK108 --barcoding -r -*
146 *o fastq,fast5 -q 0 --disable_filtering.*

147 Sequence classification

148 All sequences were BLASTed (blastn v2.6.0+) against a locally compiled database
149 consisting of the combined NCBI other_genomic and nt databases (downloaded on 13th
150 June 2018 from NCBI). Default blastn parameters were used (gapopen 5, gapextend 2),
151 and only hits with an e-value of 0.01 or less were retained. Due to the predominance of

152 short indels present in nanopore sequence data, we used the datasets basecalled in
153 January and March of 2017 to test whether changing these default penalties affected
154 the results (gapopen 1, gapextend 1). We found that these adjusted parameters did not
155 qualitatively change our results.

156 We assigned sequence reads to specific taxon levels using MEGAN6 (v.6.11.7 June
157 2018) (Huson et al. 2016). We only used reads with BLAST hits having an e-value of
158 $1x10^{-20}$ or lower (corresponding to a bit score of 115 or higher) and an alignment length
159 of 100 bp or more. To assign reads to taxon levels, we considered all hits having bit
160 scores within 20% of the bit score of the best hit (MEGAN parameter Top Percent).

161 Statistical analyses

162 Multivariate analyses were done using the software PRIMER v7 (Clarke and Gorley
163 2015). The data used in the multivariate analyses were in the form of a sample- (i.e.
164 individual rat) by-family matrix of read counts. All bacteria, rodent, and primate families
165 were removed. The majority of rodent hits were to rat and mouse, resulting from the
166 rats' own DNA (see below). The majority of the primate hits were to human sequences,
167 which probably resulted from sample contamination.

168 The read counts were converted to proportions per individual rat, by dividing by the total
169 count for each rat, to account for the fact that the number of reads varied substantially
170 among rats (Clarke et al. 2006). The proportions were then square-root transformed so
171 that subsequent analyses were informed by the full range of taxa, rather than just the
172 most abundant families (Clarke and Green 1988). We then calculated a matrix of Bray-
173 Curtis dissimilarities, which quantified the difference in the gut DNA of each pair of rats
174 based on the square-root transformed proportions of read counts across families
175 (Clarke et al. 2006).

176 We used unconstrained ordination (specifically, non-metric multidimensional scaling
177 (nMDS)) applied to the dissimilarity matrix to examine the overall patterns in the diet
178 composition among rats. To assess the degree to which the diet compositions of rats

179 were distinguishable among the three locations, we applied canonical analysis of
180 principal coordinates (CAP) (Anderson and Willis 2003) to the dissimilarity matrix. CAP
181 is a constrained ordination which aims to find axes through multivariate data that best
182 separates *a priori* groups of samples (in this case, the groups are the locations from
183 which the rats were sampled); it is akin to linear discriminant analysis but CAP can be
184 used with any resemblance matrix. The out-of-sample classification success was
185 evaluated using a leave-one-out cross-validation procedure (Anderson and Willis 2003).

186 We used Similarity Percentage (SIMPER) (Clarke 1993)) to characterise taxa that
187 distinguished between the locations. This allowed us to identify the families with the
188 greatest percentage contributions to (1) the Bray-Curtis similarities of diets within each
189 location and (2) the Bray-Curtis dissimilarities between each pair of locations.

190 Results

191 DNA sequencing and assignment of reads to taxa

192 After DNA isolation and sequencing, we obtained a total of 82,977 reads from the
193 January sequencing run and 96,150 reads from the March run. Median read lengths
194 were 606 bp and 527 bp for the January and March datasets, respectively (**Fig. 2A**).
195 These lengths are considerably shorter than other nanopore sequencing results from
196 both our and others work (Jain et al. 2016). This is most likely due to degradation of the
197 DNA during digestion in the stomach as well as fragmentation during DNA isolation
198 (Deagle, Eveson, and Jarman 2006) and sequencing library preparation. The median
199 quality scores per read ranged from 7-12 (0.80 - 0.94 accuracy) for both runs (**Fig. S1**).
200 The number of reads per barcoded rat sample varied by 10-fold for January and up to
201 40-fold in March(**Fig. 2B** and **2C**). This is primarily due to the highly variable quality of
202 DNA in each sample. However, read length and quality were similar for all samples
203 (**Fig. S1**).

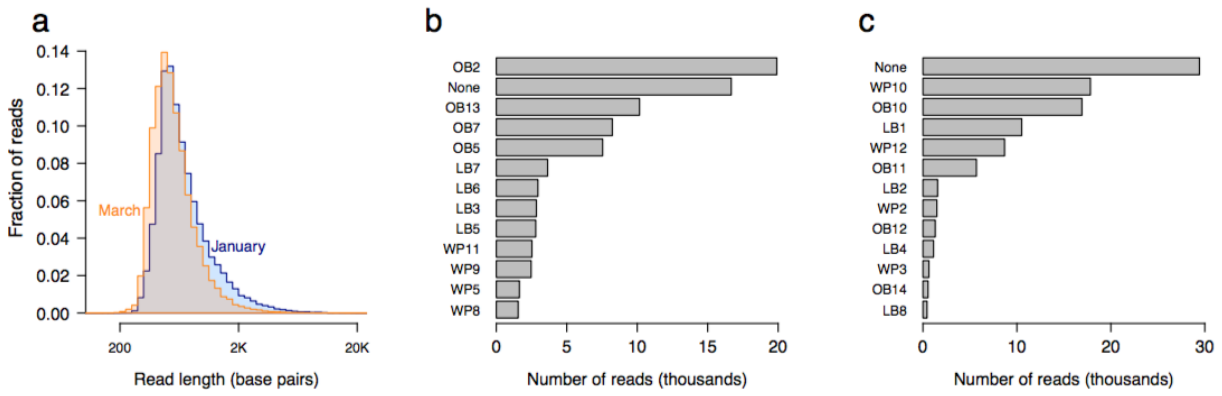

Fig. 2. Results of nanopore metagenomic sequencing of rat stomach contents. (a) Read length distribution for January and March nanopore runs. Read lengths varied between 300 and 3,000 bp. **(b) and (c) Barcode distributions for January and March runs, respectively.** We multiplexed the samples on the flow cells, using 12 barcodes per flow cell. The distribution of read numbers across barcodes was quite uneven, varying by up to 40-fold in some cases. 20% (January) and 30% (March) of all reads could not be assigned to a barcode ("None"). The inability to assign these reads to a barcode is due primarily to their lower quality.

To quantify diet contents we first BLASTed all sequences against a combined database of the NCBI nt database (the partially non-redundant nucleotide sequences from all traditional divisions of GenBank excluding genome survey sequence, EST, high-throughput genome, and whole genome shotgun (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)) and the NCBI other_genomic database (RefSeq chromosome records for non-human organisms (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)). We used BLAST as it is generally viewed as the gold standard method in metagenomic analyses (McIntyre et al. 2017). Of the 133,022 barcoded reads, 30,535 (23%) hit a sequence in the combined nt and other_genomic database at an e-value cut-off of 0.01.

224 As an initial assessment of the quality of these hits, we examined the alignment lengths
225 and e-values. We found a bimodal distribution of alignment lengths and a highly skewed
226 distribution of e-values (**Fig. 3A**). We hypothesized that many of the short alignments
227 with high e-values were false positives. We thus first filtered this hit set, only retaining
228 BLAST hits with e-values less than 1e-20. Similar quality filters have been imposed
229 previously (Srivathsan et al. 2015). A total of 22,331 hits passed this filter. Mean read
230 quality had substantial effects on the rates of BLAST success, with almost 40% of high
231 accuracy read having hits in the March dataset, as compared to 1% of low accuracy hits
232 (**Fig. 3B**).

233 To specifically assign each sequence read to a taxon, we analysed the BLAST results in
234 MEGAN6 (Huson et al. 2016). The algorithm employed in MEGAN6 assigns reads to a
235 most recent common ancestor (MRCA) taxon level. For example, if a read has BLAST
236 hits to five species, three of which have bit scores within 20% of the best hit, the read
237 will be assigned to the genus, family, order, or higher taxon level that is the MRCA of
238 those best-hit three species (Huson et al. 2007). If a read matches one species far
239 better than to any other, by definition, the MRCA is that species.

240 Of the reads from the rat stomachs with BLAST hits, 31% were assigned by MEGAN as
241 being bacterial. 55% of these were *Lactobacillus spp*. These results match previous
242 studies on rat stomach microbiomes, which have found lactobacilli to be the dominant
243 taxa (Maurice et al. 2015; Brownlee and Moss 1961; Li et al. 2017; Horáková, Zierdt,
244 and Beaven 1971). Plant-associated *Pseudomonas* and *Lactococcus* taxa were also
245 common, at 7% and 6%, respectively.

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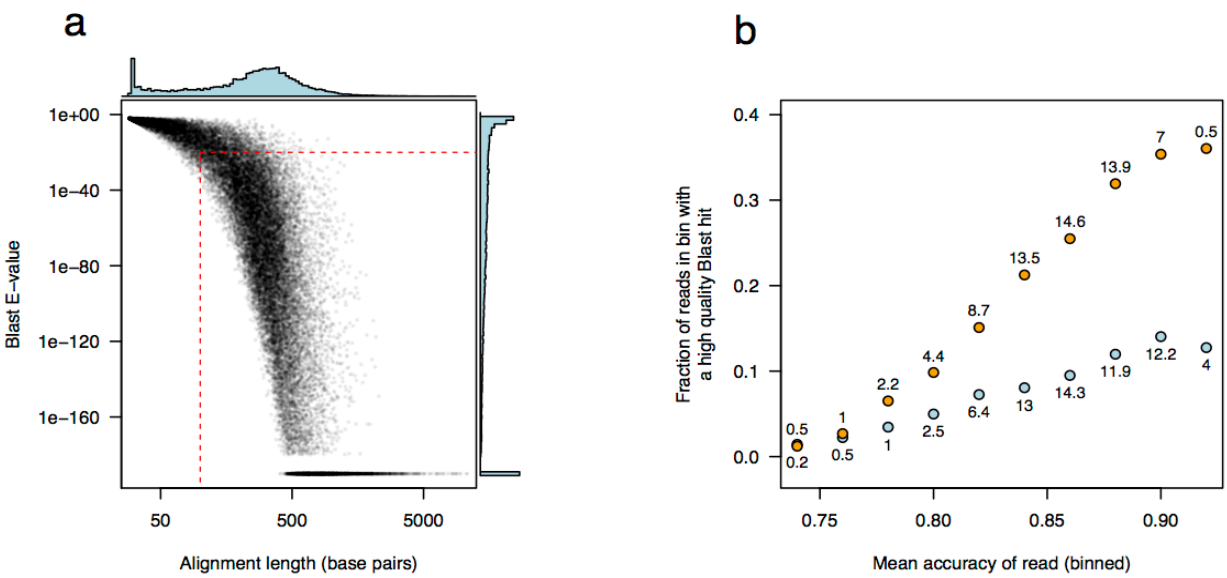


Fig. 3. BLAST hits of metagenomic reads. (a) Biplot showing the e-value and alignment length of the top BLAST hit for each read. We observed a bimodal distribution of alignment lengths and a skewed distribution of e-values. The y-axis is plotted on a log scale, with zero e-values suppressed by adding a small number (1e-190) to each e-value. The horizontal red dotted line indicates the e-value cut-off we implemented and the vertical red dotted line indicates the length cut-off (e-value < 1e-20 and alignment length of 100, respectively) to decrease false positive hits. **(b) The fraction of reads with high quality BLAST hits (e-value < 1e-20) increases as a function of read accuracy.** We binned the data according to mean read accuracy (bin width of 0.02) and calculated the fraction of reads within each bin that had a high quality BLAST hit for the January and March runs separately (blue and orange points, respectively). The number of reads in each bin is indicated above or below each point (in thousands). There is a clear positive correlation between mean accuracy and the likelihood of a high-quality BLAST hit, reaching almost 40% for very high quality reads (accuracy greater than 92.5%).

263 MEGAN assigned reads to a wide range of eukaryotic taxa. To conservatively infer
264 taxon presence, we first reclassified MEGAN species-level assignments to the level of
265 genus. However, after this, many clear false positive assignments remained (e.g. hippo
266 and naked mole rat). These matches were generally short and of low identity. To reduce
267 such false positive taxon inferences, we used information from reads assigned to the
268 genera *Rattus* (rat) and *Mus* (mouse). We inferred that the reads assigned to *Rattus*
269 (2,696 reads in total) were true positive genus-level assignments and that the reads
270 assigned to *Mus* (2,798 reads in total) were false positive genus-level assignments (and
271 not true positive *Mus*-derived reads). Although rats are known to prey on mice
272 (Bridgman et al. 2013), if this had occurred, we would expect that (1) the ratio of mouse
273 to rat reads would be higher in the subset of rats that had predated mice; (2) in those
274 same rats, the percent identity of the reads assigned to *Mus* would be higher than in
275 rats that had not predated mice. However, we found that the ratio of mouse to rat reads
276 was similar for all rats, and there was no evidence of higher percent identities for *Mus*
277 reads from rats that had higher ratios.

278 Notably, the mean percent identity values of the best BLAST hits for these two groups
279 of reads differed substantially, with *Rattus* reads having a median identity of 86.4%, and
280 *Mus* 81.0% (**Fig. 4A**). The mean percent identity for *Rattus* reads corresponds very well
281 to that expected given the mean quality scores of the reads (assuming the true
282 sequence of the read is 100% identical to *Rattus*, 86.4% identity corresponds to a mean
283 quality score of 8.7).

284 There was also a clear difference in the alignment lengths: the median ratio of
285 alignment length to read length was 0.57 for *Rattus* and 0.52 for *Mus* (**Fig. 4B**).
286 Importantly, the majority of diet items have percent identities that overlap with the
287 *Rattus* reads, and alignment length to read length ratios that exceed the *Rattus* reads,
288 suggesting that many diet taxa assignments are correct down to the level of genus.
289 However, to minimise the rates of false positive taxon assignments of diet items, we
290 implemented cut-offs based on the characteristics of the *Mus*- and *Rattus*-assigned
291 reads. For genus-level assignment, we required at least 82.5% identity and an

alignment length to read length ratio of at least 0.55. These cut-offs exclude 88% of the reads falsely assigned to *Mus*, instead assigning them correctly to one taxon level higher, the Family *Muridae*.

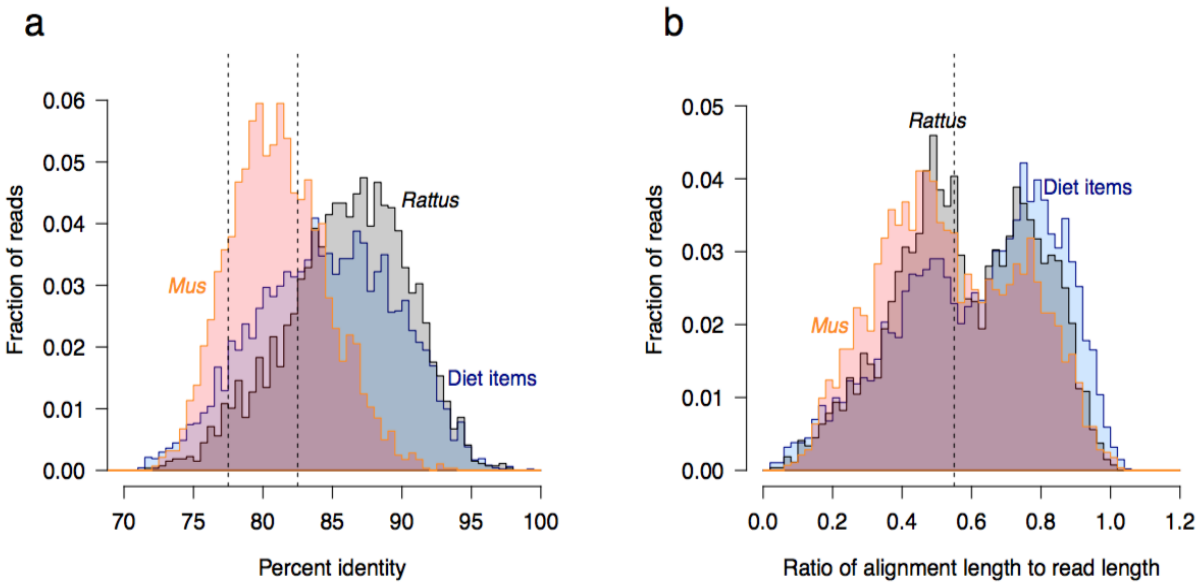


Fig. 4. Distributions of percent identity and length for alignments of reads matching *Rattus* (rat), *Mus* (mouse), and diet items. (a) Percent identity for alignments of rat (*Rattus*) and diet items is much higher than for mouse (*Mus*). Histograms are shown for the percent identity of the alignment of the top BLAST hit with the read. *Mus* matches show a clear shift to the left (lower percent identity) as compared to *Rattus* and diet items. Although different genera, *Mus* and *Rattus* are in the same family (*Muridae*). The dotted lines indicate the cut-offs that we implemented for inferring reads as belonging to a specific genus (above 82.5% identity) or family (above 77.5% identity). **(b) Ratios of alignment lengths to read lengths of rat (*Rattus*) and diet items are higher than for mouse (*Mus*).** This plot is analogous to that in (a). The dotted line indicates the cut-off that we implemented for inferring reads as belonging to a specific genus (above 0.55).

308 For family-level assignments, we required 77.5% identity, an alignment length to read
309 length ratio of at least 0.1, and a total alignment length of at least 150 bp. Using higher
310 cut-offs for the ratio of alignment length to read length excluded a large number of likely
311 true positive taxa for which only short mtDNA or rDNA database sequences were
312 present in the databases. For all other read-to-taxon assignments, we placed the read
313 at the level of Order, or used the taxon level assigned by MEGAN. Using these cut-offs,
314 16% of all reads were classified at the Genus level; 71% were classified at the Family-
315 level or below; 89% were classified at the Order-level or below; and 98% were classified
316 at the Phylum-level or below.

317 Even though we imposed these relatively stringent filters, we observed that a small
318 number of likely false positive taxa remained. Most were single reads with short
319 alignments: *Poeciliidae* (177 bp); *Salmonidae* (172 bp); *Cyprinodontiformes* (140 bp and
320 177 bp); and *Octopodidae* (151 bp). The exception to this were three reads from two
321 rats matching *Buthidae* (scorpions), which had alignment lengths of 762 bp, 664 bp, and
322 298 bp. It is unlikely these are true positives, and instead that these rats predated
323 harvestmen, a closely related sister taxon within *Arachnida*. Despite the presence of
324 these false positive taxa, to ensure we could identify the majority of taxa at high
325 resolution (i.e. family-level), we did not further increase the stringency of our filters.

326 Identification of stomach contents

327 Within each rat, a wide variety of plant, animal, and fungal families and orders were
328 discernible, ranging from two to 25 orders per rat (mean 8.7) (**Fig. 5**). Plants were the
329 primary diet item, with the largest fraction of rats consuming four predominant orders:
330 *Poales* (grasses), *Fabales* (legumes), *Arecales* (palms), and *Araucariales* (podocarps).
331 The dominance of plant matter (fruits and seeds) in rat diets has been established
332 previously (Sweetapple and Nugent 2007; Riofrío-Lazo and Páez-Rosas 2015). Animal
333 taxa made up a smaller component of each rat's diet, with *Insecta* dominating:
334 *Hymenoptera*, *Coleoptera*, *Lepidoptera* (moths and butterflies), *Blattodea*
335 (cockroaches), *Diptera* (flies), and *Phasmatodea* (stick insects). In addition

Stylommatophora (slugs and snails) were present in substantial numbers (**Fig. 6A** and **6B**). Fungi were only a small component, although several families and orders were present: *Sclerotiniaceae* (plant pathogens), *Saccharomycetaceae* (budding yeasts), *Mucorales* (pin molds), *Russulales* (brittlegills and milk-caps), and *Chytotheriales* (black yeasts). Finally, for many rats, a substantial proportion of the stomach contents were parasitic worms (primarily *Spirurida* (nematodes) and *Hymenolepididae* (tapeworms)).

Close examination of this data suggested that specific families and orders were overrepresented in the diets of rats from particular locations. For example, six out of eight rats from the native estuarine bush habitat (OB) consumed *Arecaceae*, while only one in the restored wetland area (LB) did. All three rats that consumed *Phaseanidae* were from the native estuarine habitat (OB). All five rats that consumed *Solanales* were from the restored wetland area. These patterns suggested that it might be possible to use diet components alone to pinpoint the habitat from which each rat was sampled.

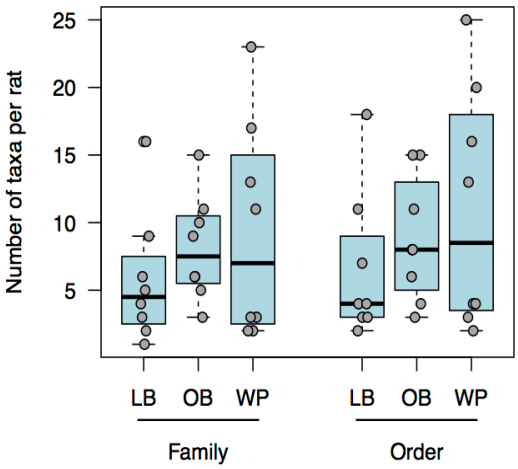


Fig. 5. Numbers of taxa in individual rats. Each boxplot indicates the range of families (left boxes) or orders (right boxes) consumed by each rat in each location (OB: Okura Bush; LB: Long Bay Park; WP: Waitakere Park). The numbers for individual rats (eight per location) are plotted in grey.

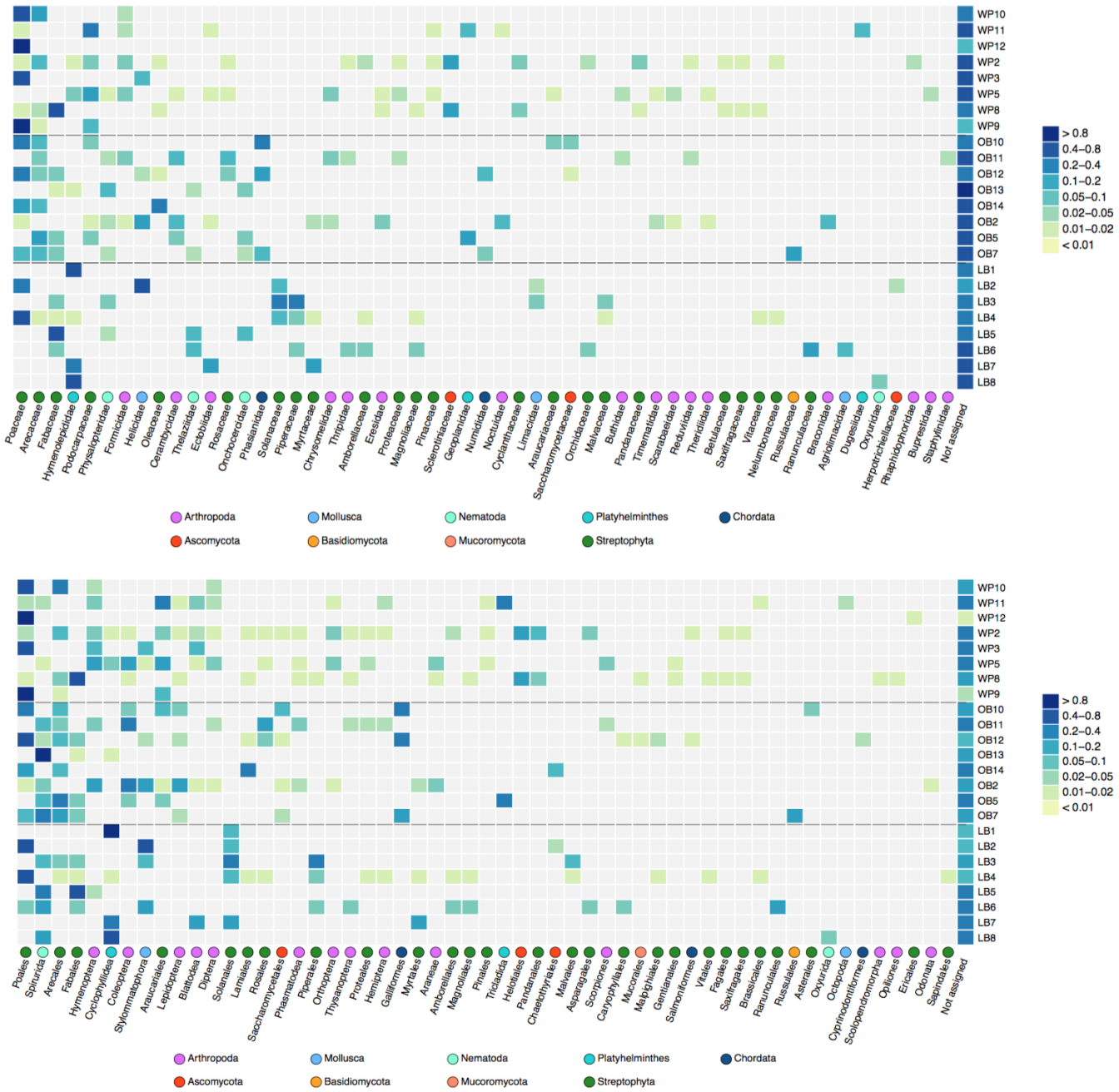


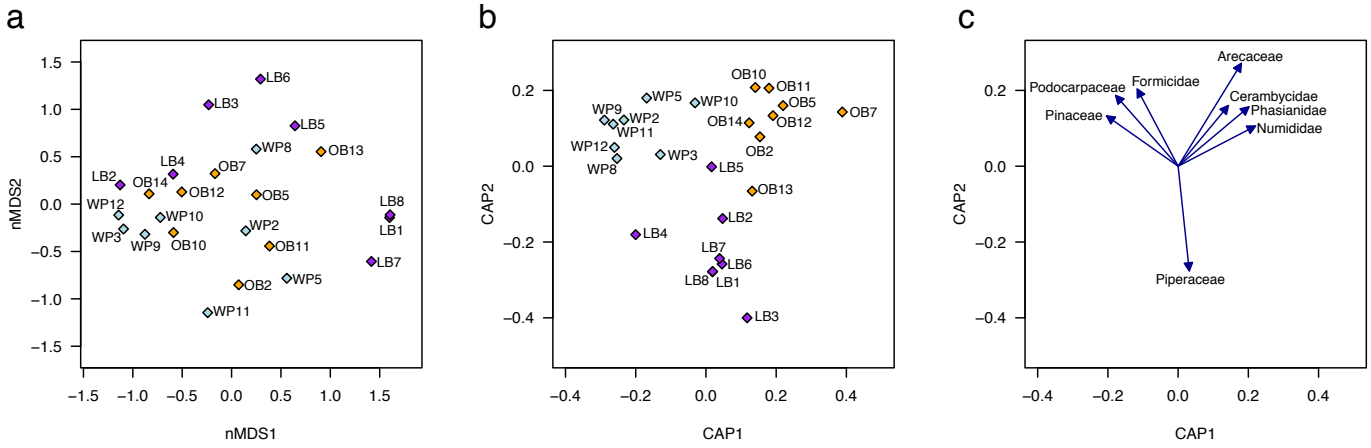
Fig. 6. Proportions of taxa in the diets of individual rats. Reads assigned to taxa at the family and order level are shown in the top and bottom panels, respectively. Each row corresponds to a single rat, with the proportions of reads for that rat assigned to each family or order indicated in shades of blue and yellow. Reads that were not assigned to a specific family or order are on the right end of the figure. The families and orders have been sorted so that the most common diet components appear on the left. Only the 55 most common families are shown. Note that the colour gradations presented on the scale are not linear.

363 nMDS and CAP analysis by location

364 In order to determine if diet composition of the rats differed consistently between
365 locations, we first performed an unconstrained analysis using nMDS on taxa assigned
366 at the family level. Using family rather than order or genus provides a balance between
367 how precisely we identify the taxon of diet item (genus, family, order), and whether we
368 assign a taxon at all. While family-level assignments are less precise than genus-level,
369 only 16% of all reads were classified at the genus level, while 71% were classified at the
370 family level.

371 The family-level unconstrained ordination (nMDS; **Fig. 7a**) showed no obvious grouping
372 of rats with respect to the locations, indicating that locations did not correspond to the
373 predominant axes of variation among the diets. However, a constrained ordination
374 analysis (CAP; **Fig. 7b**) identified axes of variation that distinguished the diets of rats
375 from different locations. The CAP axes correctly classified the locations of 19 out of 24
376 (79%) rats using a leave-one-out procedure.

377 The families that best characterised the three locations (i.e., had the greatest within-
378 location SIMPER scores) varied among locations. WP had average Bray-Curtis within-
379 location similarity of 13%; mostly attributable to *Hymenolepidae* (accounting for 51% of
380 the within-group similarity), *Solanaceae* (11%), and *Fabaceae* (11%). The average
381 similarity for OB was 21%, with the greatest contributing taxa being *Arecaceae* (33%),
382 *Poaceae* (23%), *Fabaceae* (9%), and *Phasianidae* (8%). The average similarity for WP
383 was 24%, with the greatest contributing taxa being *Poaceae* (72%) (**Table S4**). The
384 families with the largest correlations with the first two principal coordinates and thus
385 responsible for the separation between groups) were *Arecaceae*, *Podocarpaceae*,
386 *Phasianidae*, and *Pinaceae* (**Fig. 7c**).



387 **Fig. 7. Unconstrained nMDS (a) and constrained CAP (b) ordinations of the diets**
388 **of rats from three locations. Both ordinations were based on Bray-Curtis**
389 **dissimilarities of square root transformed proportions of reads attributed to each**
390 **family.** The locations were a native estuarine bush (OB, orange); a restored marine
391 wetland (LB, purple); and a native forest (WP, light blue). The CAP ordination is
392 repeated in panel **(c) with the rats omitted to show the Pearson correlations**
393 **between families and the two cap axes.** The eight families with the strongest
394 correlations are shown, indicating the taxa most strongly associated with each location.

395

396 Discussion

397 Accuracy and sensitivity

398 Here we have shown that using a simple long-read metagenomic DNA sequencing
399 approach allows rapid classification of rat diet components. We expect that this
400 technique for dietary analysis could be applied to a variety of animals and sample types,
401 including samples that require less invasive collection (e.g. fecal matter). The sensitivity
402 of this approach will likely improve as the accuracy and yield of Oxford Nanopore
403 sequencing increases, and as the diversity of taxa in genomic sequence databases
404 increases. Several aspects of the data support this.

405 First, we note that we did not find BLAST hits for the majority of reads. This is partially
406 due the relatively low accuracy of the Oxford Nanopore sequencing platform at the time
407 we preformed this analysis (approximately 87%). However, the fraction of reads yielding
408 hits in the database increased substantially for higher quality reads, approaching 40%
409 for very high-quality reads (**Fig. 3b)**. Other factors also likely reduced the numbers of
410 BLAST hits, such as the paucity of genome sequence data for many taxa. This is
411 convincingly illustrated by comparing across taxa the fraction of genomic hits to
412 mitochondrial or rDNA sequence hits: although genome data exists for only a few taxa,
413 mtDNA and rDNA sequence data are present in the database for the vast majority of
414 animal and plant genera.

415 For animals with sequenced genomes in the database, we found that only a minority of
416 reads mapped to mitochondrial or rDNA sequences (e.g. 32% of *Rattus* hits and 22% of
417 *Anoplophora* hits). For plants with sequenced genomes, the fraction of mtDNA or rDNA-
418 matching reads was even lower: between 4% (*Aegilops*) and 6% (*Triticum* and
419 *Solanum*). For genera with little or no genomic sequence, the vast majority of matches
420 were solely to mtDNA, rDNA, or microsatellite loci: 90% of *Phoenix* (date palm) hits; all
421 *Helix* (snail) and *Rhaphidophora* **(**cave weta) hits. All *Artioposthia* (New Zealand
422 flatworm) hits were to rDNA. These results indicate that for genera with no genomic
423 sequence data, we have underestimated the actual number of sequences from that
424 taxon by approximately three- to twenty-fold (for animals and plants, respectively).

425 As the species sampling of genomic databases increases (Lewin et al. 2018), the taxon-
426 level precision of this method will improve. Given the current rate of genomic
427 sequencing, with careful sampling, the vast majority of multicellular plant and animal
428 families (and even genera) will likely have at least one type species with a sequenced
429 genome within the next decade. Continued advancement in sequence database search
430 algorithms as compared to current methods (Wood and Salzberg 2014; Kim et al. 2016;
431 Nasko et al. 2018) should considerably decrease the computational workload necessary
432 to find matching sequences.

433 To decrease biases in genomic databases, some previous studies have performed
434 metagenomic classification using mitogenome data alone (Srivathsan et al. 2016; Paula
435 et al. 2016). Using such methods, they found between 0.004% and 0.008% of all
436 metagenomic reads matched mitogenomes from diet taxa. Limiting database searches
437 to mitogenomes partially ameliorates biases in terms of taxon field in terms of taxon
438 representation (i.e. most taxa will have similar levels of genomic representation in the
439 databases). However, it considerably decreases diet resolution given that for some
440 taxa, only a small percentage of sequence reads derive from the mitochondria as
441 opposed to the nuclear genome.

442 It is important to note that our interest in diet also includes resolving relative biomass
443 and relative numbers of each prey species, neither of which necessarily correlate well
444 with the amount of DNA (either mitochondrial or nuclear) purified from a sample. Even a
445 simple correction for the fraction of reads matching mitochondrial versus nuclear
446 genomes is difficult, as different plant and animal tissues differ considerably in the
447 relative amounts of mitochondrial versus nuclear DNA (e.g. leaf versus fruit).

448 Methodological advantages

449 We found that rats consumed many soft-bodied species (e.g. mushrooms, flat worms,
450 slugs, and lepidopterans) that would be difficult to identify using visual inspection of
451 stomach contents. Capturing such a wide variety of taxa would be difficult to quantify
452 using metabarcoding methods, as there are no universal 18S or COI universal primers
453 capable of amplifying sequences in all these taxa. While it might be possible to use
454 primer sets targeted at different phyla or orders, quantitatively comparing diet
455 components across these using sequences amplified with different primer sets is
456 extremely difficult due to differences in primer binding and PCR efficiency.

457 The nanopore MinION-based sequencing method used in this simple metagenomic
458 approach has several advantages. Compared to other high throughput sequencing
459 technologies (e.g. Illumina, IonTorrent, or PacBio), there is no initial capital investment

required to use the platform. On a per-sample basis, data generation is inexpensive (approximately $150 USD per barcoded sample, and approximately half this price if reagents are purchased in bulk). Library preparation and sequencing can be extremely rapid, going from DNA sample to sequence in less than two hours (Zaaijer et al. 2017). Furthermore, the sequencing platform itself is highly portable. As the cost of nanopore-based sequencing continues to decrease (both per sample and per base pair), it should become possible to use molecular methods for routine ecological monitoring of species presence or absence in field settings, without significant investment in infrastructure (Kamenova et al. 2017). Finally, we suggest that our approach of standardising the read counts by sample, followed by an optional transformation such as square root and dissimilarity-based multivariate ordination, offers a useful analytical pipeline for analysing metagenomic diet-composition data.

Conclusion

Here we have shown that a rapid long-read metagenomic approach is able to accurately characterise diet taxa at the family-level and distinguish between the diets of rats according to the locations from which they were sourced, with almost 80% out-of-sample classification success. This approach also identifies the taxa responsible for such patterns. This information may be used to guide conservation efforts toward specific areas and habitats in which native species are most at risk from this highly destructive introduced predator.

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Data Accessibility

Sequence data are available in ENA (accession number PRJEB27647)

Author Contributions

WP, JD, NF, and OS conceived the project. WP performed the stomach dissections. WP and NF optimised the genomic DNA isolation and library preparation. NF performed the nanopore sequencing. GB and OS processed and performed quality control on the sequencing data. WP and OS performed the sequence classification. WP, AS, NF, and OS analysed the data. WP, NF, AS, and OS wrote the paper, with input from all authors.

497 Supplemental Tables

498 **Table S1**. Read numbers and total base pairs for each barcode in the January
499 sequencing run.

Rat	Total reads	Total Mbp	Mean length
OB2	19.907	14.62	734
WP11	10,164	9.63	947
WP5	8237	6.78	823
LB7	7548	7.04	933
OB13	3644	3.63	995
WP9	2954	2.4	814
OB5	2850	2.06	721
WP8	2801	2.32	827
LB6	2531	1.6	632
OB7	2473	1.87	756
LB5	1641	1.16	705
LB3	1554	0.99	636
None	16673	13.01	781
Total	82,977	67.1	

500

501 **Table S2**. Read numbers and total base pairs for each barcode in the March
502 sequencing run.

Barcode	Total	Total Mbp	Mean
LB1	17,820	9.21	517
LB8	16,923	13.13	776
WP2	10,511	7.00	666
LB4	8684	4.92	567
OB11	5689	3.40	598
WP10	1563	0.99	633
OB12	1479	0.89	604
WP12	1309	0.78	596
LB2	1127	0.76	676
WP3	637	0.73	1141
OB14	541	0.37	683
OB10	435	0.24	555
None	29,432	21.33	725
Total	96,150	63.75	

503

Table S3. SIMPER analysis of family contributions to group similarities. Abbreviations: Av.Abund: average abundance; Av.Si: average similarity; Sim/SD: similarity over standard deviation; Percent.contr: percent contribution.

Family	Av.Abun	Av.Si	Sim/SD	Percent.cont	Group
Hymenolepidida	3.37	6.87	0.34	51.2	LB
Solanaceae	1.57	1.48	0.34	11.1	LB
Fabaceae	1.74	1.41	0.44	10.5	LB
Arecaceae	2.86	7.11	1	33.4	OB
Poaceae	2.87	4.82	0.55	22.7	OB
Fabaceae	1.17	1.98	0.51	9.3	OB
Phasianidae	1.79	1.67	0.34	7.9	OB
Poaceae	5.08	17.61	0.62	72.1	WP

509 **Table S4.** SIMPER analysis of family contributions to group dissimilarities. The families
510 are sorted by percent contribution to group dissimilarity. Abbreviations: Abund.Group1
511 and Abund.Group2: abundance of each family in groups 1 and 2 (see Group 1 and 2
512 columns). Av.Diss: average dissimilarity; Diss/SD: Dissimilarity over standard deviation;
513 Percent.contr: percent contribution.

Family	Abund.Group1	Abund.Group2	Av.Diss	Diss/SD	Percent.contr	Group1	Group2
Poaceae	1.95	5.08	15.15	1.04	16.74	LB	WP
Poaceae	2.87	5.08	11.29	1.26	13.78	OB	WP
Hymenolepididae	3.37	0.48	10.8	0.73	11.93	LB	WP
Hymenolepididae	3.37	0.29	9.37	0.79	10.32	LB	OB
Poaceae	1.95	2.87	8.37	1.1	9.22	LB	OB
Arecaceae	0.05	2.86	6.99	1.41	7.7	LB	OB
Arecaceae	2.86	1.31	5.92	1.29	7.23	OB	WP
Fabaceae	1.74	1.05	6.14	0.67	6.78	LB	WP
Podocarpaceae	0	2.38	5.34	0.83	5.9	LB	WP
Podocarpaceae	0.71	2.38	4.82	0.99	5.88	OB	WP
Fabaceae	1.74	1.17	4.87	0.81	5.37	LB	OB
Fabaceae	1.17	1.05	4.31	0.84	5.26	OB	WP
Phasianidae	1.79	0	4.03	0.7	4.92	OB	WP
Phasianidae	0	1.79	4.28	0.72	4.71	LB	OB
Physalopteridae	1.63	0.18	3.79	0.59	4.63	OB	WP
Physalopteridae	0.46	1.63	4.15	0.65	4.57	LB	OB
Solanaceae	1.57	0	3.95	0.65	4.37	LB	WP
Solanaceae	1.57	0	3.56	0.67	3.92	LB	OB
Helicidae	0.87	0.47	3.46	0.52	3.82	LB	WP
Oleaceae	1.06	0.25	3.09	0.44	3.77	OB	WP
Arecaceae	0.05	1.31	3.4	0.65	3.75	LB	WP
Piperaceae	1.42	0	3.36	0.65	3.71	LB	WP
Helicidae	0.87	0.82	3.33	0.58	3.66	LB	OB
Cerambycidae	1.41	0.18	2.99	0.77	3.65	OB	WP
Cerambycidae	0	1.41	3.07	0.74	3.38	LB	OB
Oleaceae	0	1.06	3.06	0.41	3.37	LB	OB
Piperaceae	1.42	0	3.04	0.67	3.35	LB	OB
Onchocercidae	0.39	1.04	3.02	0.77	3.33	LB	OB
Geoplanidae	0.8	0.48	2.64	0.49	3.22	OB	WP
Thelaziidae	0.9	0.64	2.85	0.77	3.14	LB	OB
Helicidae	0.82	0.47	2.54	0.61	3.1	OB	WP
Onchocercidae	1.04	0	2.49	0.66	3.04	OB	WP
Formicidae	0.44	1.17	2.39	1.04	2.92	OB	WP

Sclerotiniaceae	0	1.28	2.63	0.57	2.91	LB	WP
Sclerotiniaceae	0	1.28	2.3	0.57	2.81	OB	WP
Formicidae	0	1.17	2.46	0.94	2.72	LB	WP
Rosaceae	0.92	0.3	2.13	0.58	2.61	OB	WP
Ectobiidae	0.63	0.34	2.24	0.5	2.48	LB	WP
Thelaziidae	0.9	0	2.13	0.56	2.36	LB	WP
Geoplanidae	0	0.8	2.04	0.37	2.25	LB	OB
Rosaceae	0	0.92	1.99	0.5	2.19	LB	OB
Numididae	0.78	0	1.68	0.56	2.05	OB	WP
Myrtaceae	0.66	0	1.8	0.38	1.99	LB	WP
Thelaziidae	0.64	0	1.53	0.51	1.86	OB	WP
Ranunculaceae	0.66	0	1.36	0.37	1.5	LB	WP

Datafile S1. Table of read BLAST hits and assigned MEGAN taxa with reads reclassified at the family or order level by filtering on read length to alignment length ratio and percent identity.

Datafile S2. Table of read BLAST hits and assigned MEGAN taxa with no filters applied.

Supplemental Figures

Fig S1. Biplots of read lengths and qualities for each barcode in the January and March runs.

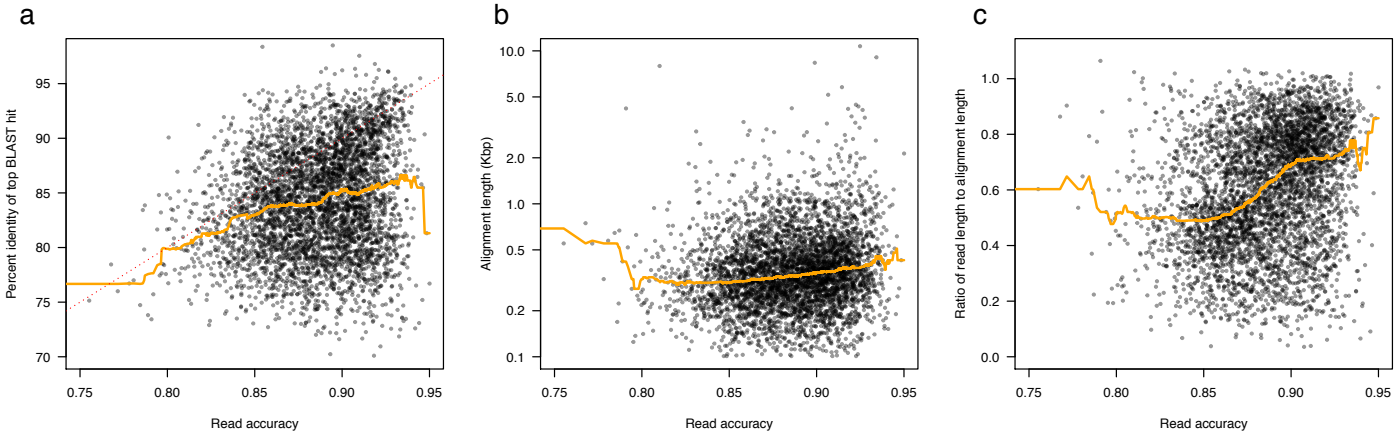

Fig S2. Correlation of read accuracy with alignment characteristics. **(a)** Read accuracy is positively correlated with the percent identity of the top BLAST hit. Points show a subsample of reads; orange line indicates a running median; red dotted line is the y=x line, which is expected if accuracy corresponds exactly to percent identity. **(b)** Read accuracy and alignment length show no relationship. **(c)** Read accuracy and the ratio of read length to alignment length are positively correlated.

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